

Copper Tolerance of *Trichoderma koningii* Tk10

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Abstract: Copper (Cu) is an essential micronutrient for all living organisms, serving as a cofactor for numerous enzymes. However, excessive copper concentrations can be harmful. To investigate copper tolerance in fungi, a copper-tolerant strain was isolated from soil and identified as *Trichoderma koningii* Tk10, with optimized culture conditions being established. Additionally, copper-related genes were analyzed through whole-genome sequencing. The results indicated that Tk10 exhibits a maximum copper tolerance of 5.4 mmol/L and a maximum adsorption rate of 51.5% under optimal cultivation conditions. Whole-genome sequencing revealed six genes associated with copper tolerance, including one superoxide dismutase gene, one peroxidase gene, and three catalase genes linked to copper stress. Furthermore, the enzyme activities of the catalase, superoxide dismutase, and peroxidase significantly increased, reaching levels that were 8.02, 4.12, and 3.88 times higher than those observed in the control group, respectively. A real-time quantitative PCR analysis indicated that three of these genes were significantly upregulated in response to copper stress. The findings of this study provide valuable insights into copper tolerance in filamentous fungi.

Keywords: copper tolerance; *Trichoderma koningii*; genome-wide; antioxidant enzymes

1. Introduction

Copper is essential for growth and development, serving as a critical cofactor in the superoxide dismutase of both CU and ZN, which detoxify oxygen radicals in cytoplasm. Additionally, copper plays a crucial role in stabilizing protein conformation by binding to various proteins [1]. However, excessive copper can be detrimental to health, as high concentrations of copper ions may disrupt the normal conformation and function of proteins [2]. Due to this dual role, all life forms have evolved diverse mechanisms to maintain copper homeostasis, including copper chelation, translocation, and efflux [3].

Currently, the primary methods for addressing heavy metal contamination include ion exchange, electrolysis, membrane separation, chemical precipitation, and bioremediation. Among these, bioremediation is extensively utilized due to its low energy consumption, economic viability, environmental friendliness, and ease of operation [4]. A variety of bacteria, fungi, and plants with significant bioremediation potential have been documented [5]. Due to the advantages of rapid growth, strong stress resistance, larger cell-to-surface ratios, and high biomass, fungi have significant advantages in bioremediation applications [6]. Numerous fungi have demonstrated the ability to remove different types of pollutants. For example, six fungal strains were selected based on their tolerance and high capability in regard to accumulating heavy metals, achieving a copper bioaccumulation of 84% (*Mortierella* sp. strain LG01), 49% (*Clonostachys* sp. strain CQ23), and 48–77.5% (*Trichoderma* sp. strain LM01A) [7]. Fungi detoxify copper ions in various ways, such as extracellular chelation, degradation, intracellular binding, and excretion. Extracellular



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chelation is characterized by the secretion of fungal metabolites, such as siderophores, organic acids, and enzymes, which facilitate the precipitation and immobilization of metals [8]. The cell wall also acts as an extracellular barrier to preclude metal toxicity in fungi. Metallothioneins (MTs), as cysteine-rich proteins incorporating metal ions as cofactors, play a crucial role in intracellular binding [9]. They sequester free cytosolic ions that can be released back into the cellular environment under metal-deficient conditions [10]. Copper homeostasis pathways have been extensively studied in yeast. The process begins with the adsorption of copper ions entering the cell. Once inside, copper (II) ions are reduced to copper (I) by the action of the FRE1/2 gene and subsequently transported into the cell by the CTR1/3 copper transporter gene. Subsequently, copper chaperone proteins bind specifically to copper and facilitate its delivery to various organelles [11].

Trichoderma spp. are fungi with a global distribution that flourish across diverse ecological niches; they are noted for their swift growth and robust resistance to numerous environmental pollutants, factors which render them optimal for bioremediation purposes [12]. Additionally, these organisms demonstrate resistance to various agrochemicals, polyaromatic hydrocarbons, and heavy metals, including cadmium, copper, mercury, zinc, and lead [13].

In this work, we used *Trichoderma koningii* strains (specifically Tk10) that were previously isolated from soil to assess their tolerance to copper. We enhanced the copper tolerance of the strains by single-factor optimization and investigated their capacity to adsorb copper. Furthermore, we conducted a whole-genome sequencing analysis of genes related to copper tolerance.

2. Materials and Methods

2.1. Strain

Trichoderma koningii Tk10 was isolated from forest field in Nanchang, Jiangxi. The strains were identified by morphological observations and molecular analysis of translation elongation factor 1-alpha (*tef1*). The strain conidia were preserved at -20°C in a 25% glycerol solution.

2.2. Isolation and Screening of Copper-Resistant *Trichoderma* Strains

Soil samples were collected from forest field in Nanchang City, JiangXi Province, China. Isolation of the fungal strains was carried out following the normal procedure. In short, strains were inoculated in Petri plates containing a PDA medium (200 g/L potato, 20 g/L glucose, 15 g/L agar, pH 7.0, supplemented with 1% streptomycin) incubated at 28°C until fungi colonies were visible. Then, they were subcultured to obtain pure cultures. All strains were stored at 4°C for further analysis. The pure cultures were inoculated in a screening medium (5 g/L peptone, 10 g/L glucose, 1 g/L KH_2PO_4 , 0.5 g/L MgSO_4 , 0.5 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 15 g/L agar) to obtain the copper-tolerant strains.

2.3. Morphological and Molecular Analysis

Mycelial plugs (0.5 cm in diameter) of the selected strains were transferred to the edge (0.5 cm from the margin) of PDA Petri dishes (8.5 cm in diameter) and cultured at 28°C for 72 h. The colony diameter was measured every 12 h. After the strains produced conidia, they were observed and photographed under an optical microscope, followed by a preliminary identification of the taxonomic group to which each strain belongs. The molecular analysis was conducted in two phases. First, genomic DNA was extracted using a commercial kit (B518262, Sangon Biotech, Shanghai, China), which was utilized for the amplification of translation elongation factor 1-alpha (*tef1*) according to standard protocols (primers *tef1*F CATCGAGAAGTTCGAGAAGG/*tef1*R AACTTGCAGGCAATGTGG).

Second, a phylogenetic tree was constructed using MEGA 7.0 based on highly homologous sequences obtained from NCBI BLAST alignment.

2.4. Single Factor Optimization of Culture Conditions

For higher copper tolerance, optimizing the culture medium in regard to elements such as carbon source, nitrogen source, initial pH value, and cultivation time is advised. The basic fermentation medium was 5 g/L peptone, 10 g/L glucose, 1 g/L KH_2PO_4 , 0.5 g/L MgSO_4 , and 0.5 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 15 g/L agar. In carbon source optimization, the selected strain was cultured on a basic medium with different carbon sources (including lactose, glucose, saccharose, fructose, mannitol, glycerin, maltose and soluble starch), but other sources still remain to be tested. In the optimization of nitrogen sources, the ideal carbon source was selected while keeping other components constant. The nitrogen source, which included tryptone, casein peptone, NaNO_3 , NH_4 , $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 , was the sole variable in our study. Similarly, the pH value had different gradients (including 5, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0). The assay was performed in triplicate.

2.5. Copper Bioremediation Properties

The strain was cultured in a shaking flask containing an optimal medium at 28 °C and 200 rpm. Following incubation, the mycelia were harvested, thoroughly washed with deionized distilled water, and dried on filter paper at 60 °C for 24 h until a constant weight was achieved. Subsequently, the weight of the mycelia was recorded. The residual concentration of Cu^{2+} was measured using an atomic absorption spectrophotometer (Shimadzu, Shimadzu Enterprise Management (Tianjin, China) Co., Ltd.) with an air–acetylene flame. The assay was performed in triplicate.

2.6. Measured Enzyme Activities

Three mycelium cakes (0.5 cm) were inoculated into optimal medium containing 2 mmol/L copper and were incubated at 28 °C with shaking at 180 rpm for 96 h. Following incubation, the mycelia were harvested and thoroughly washed with deionized distilled water. A total of 0.3 g of mycelium was transfer to a centrifuge tube, and 0.5 mL of protein extraction buffer was added. The resultant mixture underwent ultrasonic disruption at 60% intensity for a total duration of 60 s, with interruptions of 2 s every 4 s. Subsequently, the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was collected as the crude enzyme solution. The activities of catalase (CAT), peroxidase (POD), and dismutase (SOD) enzymes were determined using the Solarbio protease activity assay kits (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China. (<http://www.solarbio.com> (accessed on 11 January 2025))). The assay was performed in triplicate.

2.7. Whole-Genome Sequencing

Whole-genome sequencing of Tk10 was conducted using the PacBio RS II sequencing platform at Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). A library with an insert size of 325 bp was constructed for this platform. A total of 36,945 reads, with approximately 135-fold coverage (approximately 5412 Mb), were obtained. The sequences were assembled into 1153 contigs using SOAPdenovo software2.

2.8. Comparison and Analysis of the Proteins

A phylogenetic tree was constructed using a neighbor-joining method implemented in MEGA version 7.0 (A bootstrap analysis of 1000 replicates was used) using the full-length amino acid sequences of the selected genes (including seven genes of Tk10). The protein motif analysis was carried out on the Multiple Em for Motif Elicitation (<https://meme-suite.org/meme/tools/meme> (accessed on 11 January 2025)).

2.9. Quantitative RT-PCR

The strain was cultured in a shaking flask containing the optimal medium at 28 °C and 200 rpm for 48 h. Subsequently, copper was added at a final concentration of 2 mM with an untreated control group for comparison. After an additional 12 h cultivation period, the mycelium was filtered to extract RNA. Total RNA was isolated using the Trizol method (Invitrogen); cDNA was then synthesized using a TaKaRa PrimeScript™ II First Strand cDNA synthesis kit (Baori Medical Biotechnology Co., Ltd., Beijing, China. (<https://www.takarabiomed.com.cn/> (accessed on 11 January 2025))). Quantitative real-time PCR (qRT-PCR) was performed using the TaKaRa PrimeScript RT-PCR kit (DRR081A) on Bio-Rad CFX connect. A total of 25 µL was used in the PCR system containing 12.5 µL of Ex Taq, 1 µL of forward primer, 1 µL of reverse primer, 1 µL of cDNA template, and 9.5 µL of deionized water. The PCR reaction conditions used were as follows: preheating at 95 °C for 30 s; 35 cycles of 95 °C for 20 s; 55 °C for 30 s; and 72 °C for 20 s. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene (119 bp) was set as the internal reference. The expression levels were relative to the wild-type (WT) strain and calculated using the following equation:

$$\text{Relative mRNA} = 2^{-[(C_{tt} - C_{tti}) - (C_{tw} - C_{twi})]} \quad (1)$$

where C_{tt} is C_t value of the treatment, C_{tti} is C_t value of the internal reference of the treatment, C_{tw} is C_t value of the control, and C_{twi} is the C_t value of the internal reference of the control. The assay was performed in triplicate.

2.10. Statistical Analysis

All data were analyzed through a one-way analysis of variance (ANOVA) in the R programming language (R version 4.3.1). Statistical differences were established with a p value ≤ 0.05 (differentiate the variations in the figure using the letters a, b, c et al.). All data are presented as mean $\pm$ standard deviation (mean $\pm$ S.D.).

3. Results

3.1. Strain Isolation and Identification

In summary, 115 *Trichoderma* spp. strains were tested for their copper tolerance ability. Among these, one strain which showed a high ability in regard to copper tolerance was identified as *Trichoderma koningii* Tk10 isolate. The morphological property of strain Tk10 was observed using a microscope (Figure 1A–C). After incubation for 72 h at 28 °C, the colony diameter of Tk10 was 6.42 cm and the first green conidia appearing on the colony surface. Conidiophore branching occurred at angles less than 90° (Figure 1C). The morphological identification of Tk10 was supplemented by sequencing the *tef1* gene (Figure 1E). BLASTn analysis revealed that the sequence showed 99.1 similarity with *T. koningii* strain S273 (KJ665531). Through the integration of morphological characteristics and molecular biology identification, the strain was conclusively identified as *T. koningii*, named as Tk10 (GenBank number OR076447).

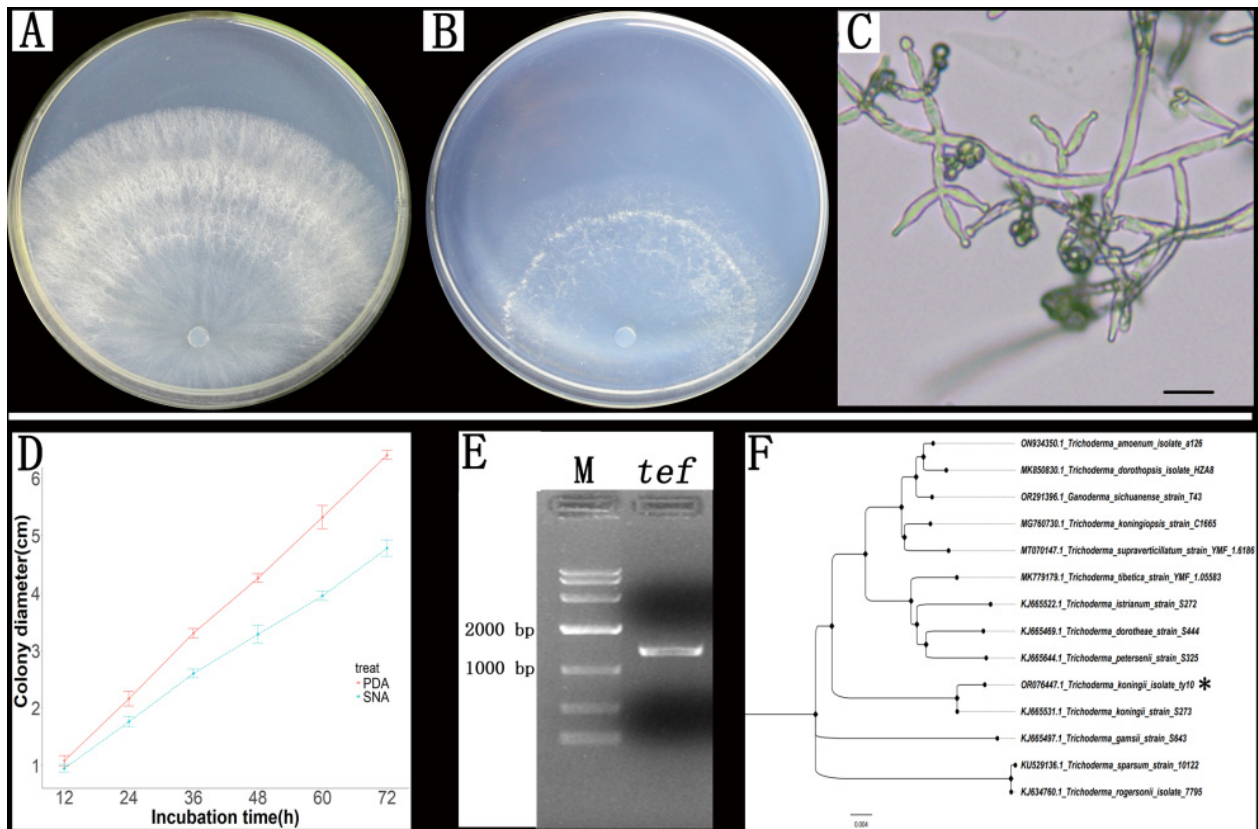


Figure 1. (A) medium; (B) the morphology of the SNA medium; (C) conidiophores and phialides, The scale is 10 μ m; (D) growth rate; (E) amplification of the *tef* gene; (F) phylogenetic tree, * highlights Tk10.

3.2. Optimization of Growth Conditions of Tk10 to Increase Copper Tolerance

In order to improve copper tolerance, the fermentation conditions, including the carbon source, nitrogen source, and initial pH, were optimized (Figure 2). The strain demonstrates the highest growth rate when glucose is employed as the carbon source in a medium with 2 mM copper. Additionally, its growth rate exceeds the average even with the absence of copper in the medium. Therefore, glucose is selected as the primary carbon source (Figure 2A). Following the selection of glucose as the optimal carbon source, the impact of various nitrogen sources was investigated. When casein peptone serves as the nitrogen source, the strain shows the highest growth rates in both copper-free and copper-containing media, leading to its selection as the preferred nitrogen source (Figure 2B). It was also observed that the pH value had a significant impact on copper tolerance. At a pH of 7.0, the strain exhibits robust growth in the medium containing copper, and it surpasses the average growth rate even in the copper-free medium. Given the potential for metal ion precipitation and its notable impact on fungal growth under alkaline conditions, pH 7.0 is selected as the optimum level (Figure 2C). Therefore, the optimal cultivation conditions are established as follows: glucose acts as the carbon source, casein peptone as the nitrogen source, and the initial pH is maintained at 7.0. Under these conditions, the strain demonstrates a maximum copper tolerance of 5.4 mM.

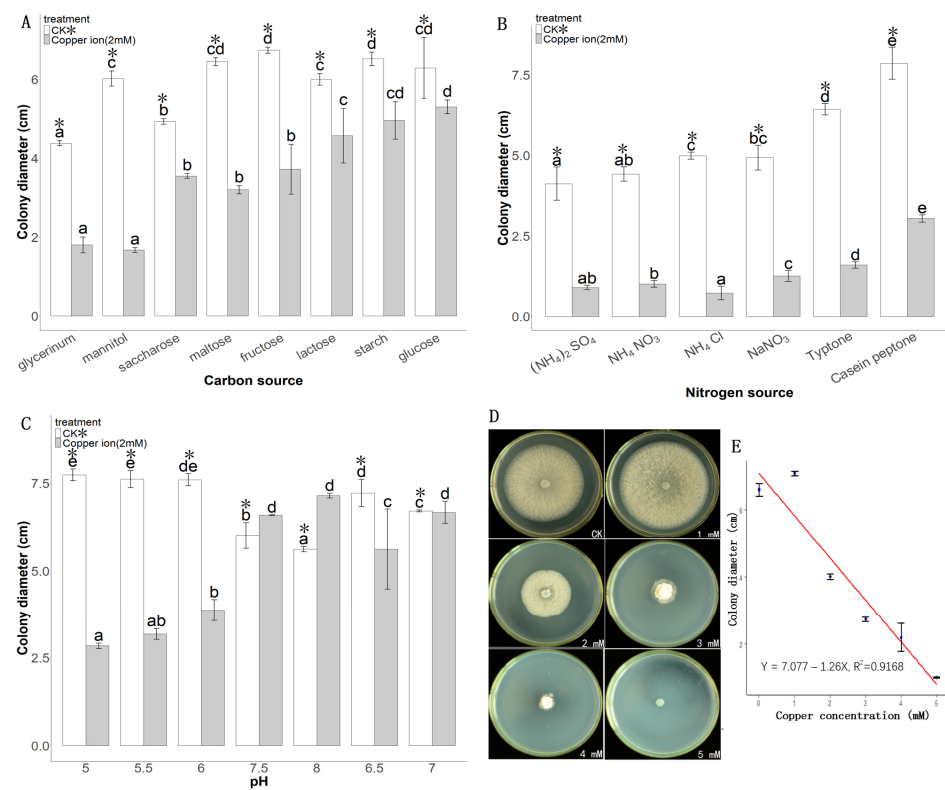


Figure 2. Copper tolerance of Tk10. (A) carbon sources; (B) nitrogen sources; (C) initial pH; (D) copper tolerance under the optimal cultivation conditions; (E) copper concentration. The letters “a”, “b”, and others indicate differences, while distinct letters denote statistically significant differences.

3.3. Copper Bioaccumulation

Copper adsorption represents one of the key mechanisms by which *Trichoderma* species tolerate heavy metals. Following a five-day shaking flask cultivation under optimal conditions, the strain’s copper adsorption capacity was assessed. The results indicate that, as the copper concentration increases, the strain’s growth rate progressively declines (Figure 3). The maximum copper adsorption capacity, achieving a removal rate of 51.5%, was recorded at a copper concentration of 1 mM, after which it began to diminish. Simultaneously, as the copper concentration increased, the coloration of the biomass distinctly deepened, indicating a significant accumulation of copper ions within the mycelium.

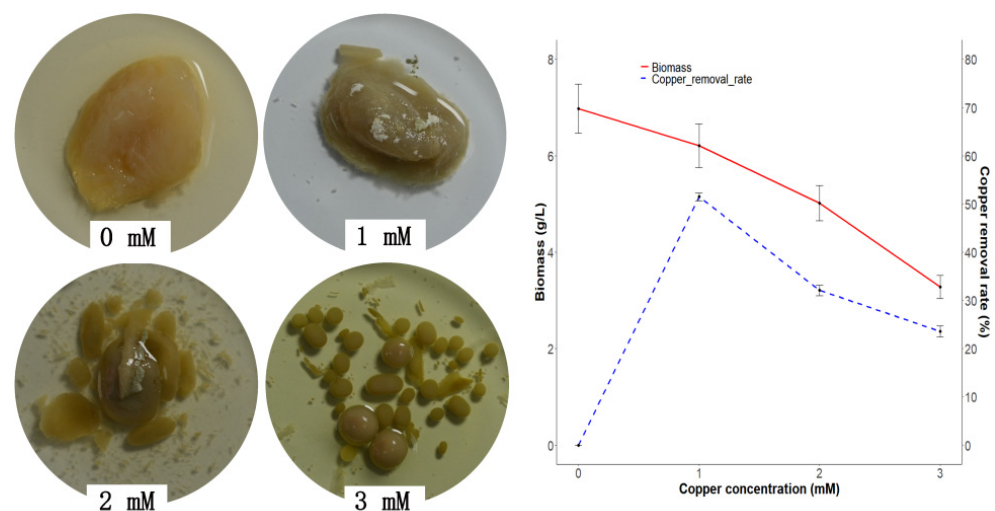


Figure 3. Copper bioaccumulation of Tk10.

3.4. Enzyme Activity Analysis of Catalase (CAT), Peroxidase (POD), and Superoxide Dismutase (SOD) Under Copper Stress

The key protein classes involved in this mechanism include CAT, POD, and SOD. After 96 h of growth under 2 mM copper stress, the activities of these three enzymes showed a significant increase. Specifically, CAT enzyme activity was 8.02 times higher than in the control, while POD enzyme activity was 3.88 times higher and SOD enzyme activity was 4.12 times higher (Figure 4).

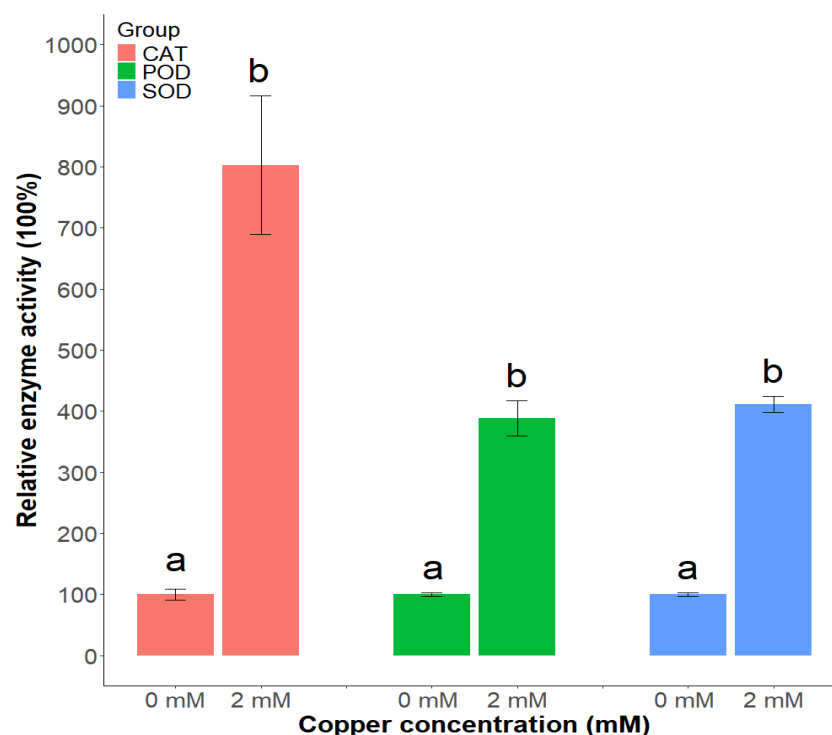


Figure 4. Relative enzyme activity of CAT, POD and SOD. The letters “a”, “b”, and others indicate differences, while distinct letters denote statistically significant differences.

3.5. Genome Sequencing

To investigate the molecular mechanism, we sequenced the whole genome of Tk10 to search the copper tolerance-related genes. The complete genome of Tk10 is 37.2 Mb and contains 6992 predicted genes (Table 1). These genes were included in 25 functional categories (according to KOG), including RNA processing and modification (112 genes), chromatin structure and dynamics (33 genes), energy production and conversion (190 genes), cell cycle control (60 genes), amino acid transport and metabolism (172 genes), nucleotide transport and metabolism (48 genes), and carbohydrate transport and metabolism (96 genes) (Figure 5). The complete genome sequences of Tk10 have been deposited in GenBank database with accession number PRJNA880568.

Table 1. General genome features of *Trichoderma koningii* Tk10.

Feature	Value
Raw Data (Mb)	7503
Filtered Reads (%)	11.67
Clean Data (Mb)	6628
Genome size (Mb)	37.2
GC content (%)	48.75
Predicted genes	6992

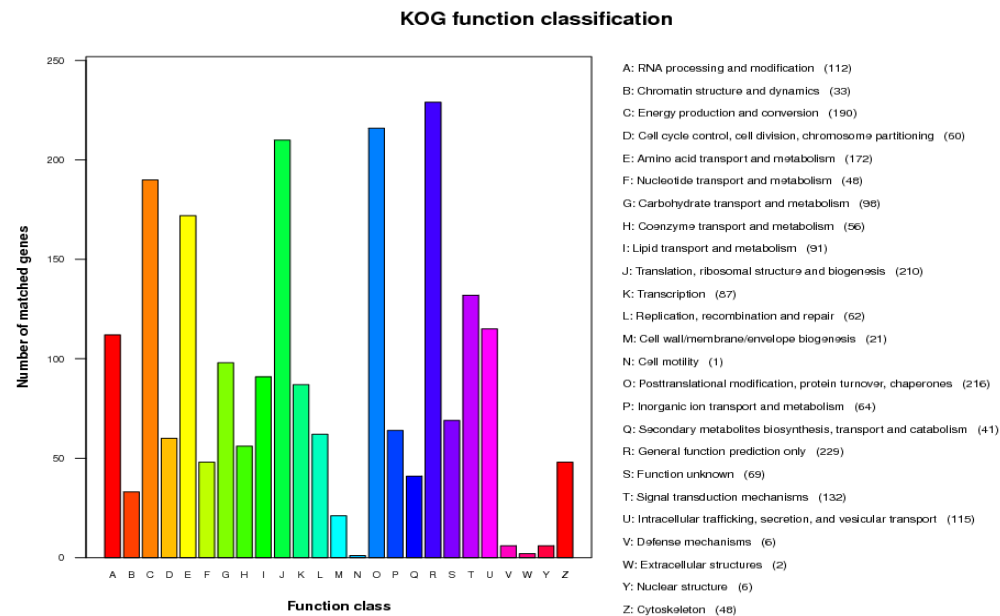


Figure 5. KOG pathway annotation of Tk10.

3.6. Copper-Related Genes Prediction

To identify copper tolerance-related genes, we retrieved 12 fungal catalase (CAT) protein sequences, 11 fungal peroxidase (POD) protein sequences, and seven fungal superoxide dismutase (SOD) protein sequences from the Protein Data Bank (PDB). These sequences were then used to query the Tk10 proteins database through the basic local alignment search tool. Our results revealed that four predicted proteins exhibited sequence similarity with fungal CAT, one predicted protein showed similarity with fungal POD, and one predicted protein displayed similarity with fungal SOD. The gene length extended from 1010 to 2463 bp with 1–10 introns. The protein length extended from 154 to 737 amino acids (Table 2).

Table 2. The predicted copper tolerance-related genes of Tk10.

Gene ID	Function	Subject ID	Gene Length (bp)	Intron	Protein (aa)
<i>tk10A1437</i>	Catalase	gi 358392193 gb EHK41597.1	2267	1	737
<i>tk10A6051</i>		gi 358396285 gb EHK45666.1	2463	10	493
<i>tk10A2372</i>		gi 358395560 gb EHK44947.1	1863	5	504
<i>tk10A4837</i>		gi 358392347 gb EHK41751.1	1864	3	533
<i>tk10A5165</i>	Peroxidase	gi 358395813 gb EHK45200.1	1122	1	350
<i>tk10A2641</i>	Superoxide dismutase	gi 358398082 gb EHK47440.1	1010	4	154

3.7. Protein Structural Analysis

To systematically analyze protein structure diversity and predict their functions, we employed MEME software to examine full-length protein sequences for the identification of conserved motifs. Phylogenetic relationships and motif analyses indicate that superoxide dismutase (SOD) proteins, including Tk10A2641, possesses four motifs, which are shared with the four previously mentioned proteins (Figure 6A). Regarding peroxidase (POD), the protein Tk10A5165 contains four motifs (Figure 6B). For catalase (CAT), all four proteins contain four motifs (Figure 6C).

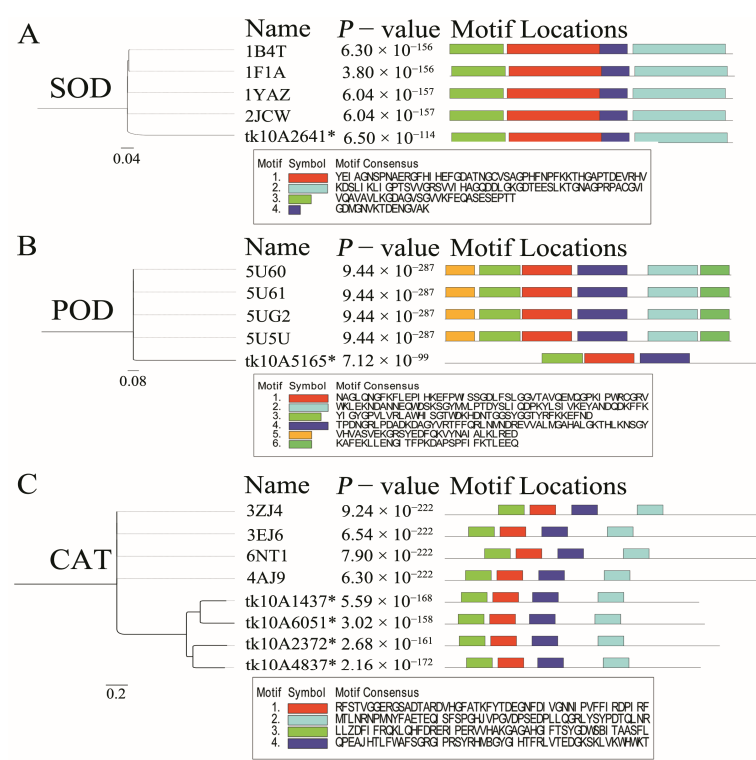


Figure 6. The phylogenetic relationships and motifs of SOD (A), POD (B), and CAT (C).

3.8. Expression Profiles of SOD, CAT and POD Genes Under Copper Stress

The accumulation of heavy metals can result in the upregulation of numerous proteins, leading to the mitigation of cell toxicity. To confirm the expression of these genes, a quantitative PCR was conducted to compare expression levels under copper stress. This analysis revealed that the SOD gene *tk10A2641* displayed the highest expression level under copper stress. The other two genes, the CAT gene *tk10A4837* and the POD gene *tk10A5165*, also exhibited elevated expression levels. However, the expression levels of the remaining three CAT genes (*tk10A1437*, *tk10A2372*, and *tk10A6051*) did not show a significant difference (Figure 7).

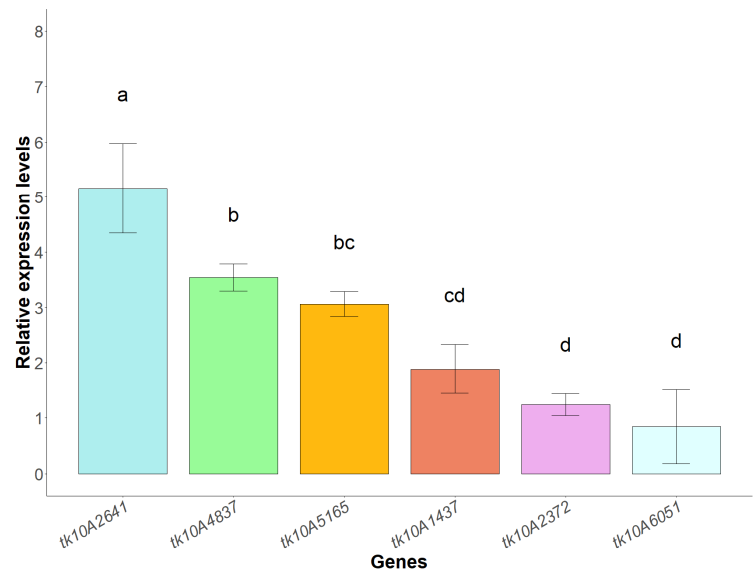


Figure 7. Relative expression profiles of SOD, CAT, and POD genes under copper stress. The letters “a”, “b”, and others indicate differences, while distinct letters denote statistically significant differences.

4. Discussion

The use of fungal biomasses for biosorption has attracted the attention of many researchers due to their numerous advantages. *Trichoderma* spp. represent a significant group of biocontrol agents for plant diseases and have also been shown to be environmental microorganisms capable of degrading and transforming pollutants [14]. In this study, we isolated and screened a strain of *Trichoderma koningii* (Tk10) with high heavy metal tolerance from soil samples. Following the optimization of cultivation conditions, the maximum copper tolerance of the strain was determined to be 5.4 mM (320 mg/L, Figure 2). Furthermore, it also showed a maximum adsorption rate of 51.5% under optimal cultivation conditions (Figure 3). Gordana investigated the copper concentration of 23 agricultural soil samples and found that the highest concentration of copper was 19.38 mg/kg [8]. Babu et al. (2014) categorized *T. virens* as a metal-tolerant fungus because of its potential to tolerate heavy metals such as Pb, Cd, Cu, As, and Zn from liquid media comprising 100 mg/L of heavy metals [15]. Therefore, our strain exhibits a high tolerance in regard to copper and has the potential for application in the remediation of copper contamination in soil. Numerous studies have shown that microbial populations in copper-polluted environments develop the ability to adapt to high levels of contamination. However, our understanding of the mechanisms underlying copper resistance in filamentous fungi remains limited. One potential mechanism may involve the activation of reactive oxygen species (ROS). Antioxidant enzymes may protect fungi from the deleterious effects of ROS and play an important role in Cu tolerance in *Trichoderma* [16]. SOD is an important endogenous antioxidant enzyme that acts as a component of first line defense systems against reactive oxygen species [17]. CAT is a common antioxidant enzyme that uses either iron or manganese as a cofactor and catalyzes the degradation or reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen, consequently completing the detoxification process imitated by SOD [18]. In the current study, we identified one superoxide dismutase (SOD) gene, one peroxidase (POD) gene, and four catalase (CAT) genes (Table 2). A gene structure analysis revealed that the number of exons varied from one to ten. To better understand the genes' role against the copper stress, the mRNA expression levels were predicted. Under 2 mM copper stress, the activities of the enzymes catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) significantly increased, reaching levels that were 8.02, 4.12, and 3.88 times higher than those observed in the control group, respectively (Figure 4). Furthermore, the expression of the SOD gene Tk10A2641, the POD gene Tk10A5165, and the CAT gene Tk10A4837 was found to be upregulated, indicating that these genes may play a role in alleviating heavy metal toxicity. Research on the mechanisms of copper tolerance in fungi is limited, particularly concerning the tolerance mechanisms of *Trichoderma* species. In this study, to identify copper tolerance-related genes, we conducted a whole-genome sequencing analysis and identified three potential genes associated with copper tolerance. The findings provide a solid theoretical basis for the bioremediation applications of heavy metals.

5. Conclusions

Numerous reports have demonstrated that, in heavy-metal-polluted environments, microbial populations develop the ability to adapt to the high contamination levels [19]. In this study, we isolated a strain of *Trichoderma koningii* Tk10 with high copper tolerance from soil samples. Under optimal culture conditions, the strain demonstrated a copper tolerance of 5.4 mM and a copper adsorption rate of 51.5%. Copper has been utilized for many years as an antimicrobial agent in various applications; however, it is often applied in doses that significantly impact the environment. A comprehensive assessment of the mechanisms underlying fungal copper resistance is essential for identifying new and

effective alternatives for remediation of heavy metal copper pollution. We assessed the enzyme activities and corresponding gene expression levels under copper stress, and the results indicated that both enzyme activity and gene expression increased proportionally. Future research will focus on elucidating the mechanisms underlying copper tolerance in this strain using techniques such as gene knockout.

Author Contributions: Designed the experiments, participated in genome comparison of selected genes, and contributed to the writing of the manuscript, K.F. and L.F.; cultivation, DNA extraction and writing—original draft preparation J.J.; software and writing, X.Q. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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